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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Roland Valdes Jr. et al. Art Unit : 1648
Serial No. : 09/503,559 Examiner : U. Winkler
Filed : February 11, 2000
Title : DIHYDROOUABAIN-LIKE FACTOR AND DIAGNOSTIC & THERAPEUTIC COMPOSITIONS AND METHODS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

I, Roland Valdes, Jr., hereby declare as follows:

(1) I am one of the co-inventors of the above-identified patent application. I currently hold the position of Professor in the Department of Pathology and Laboratory Medicine, Senior Vice-Chairman Academic Affairs and Research, and Director of Clinical Chemistry and Toxicology at the University of Louisville.

(2) Dh-OLF (a compound isolated from mammals) is structurally different than dihydroouabain-B (a compound isolated from plants). Although we used similar HPLC techniques in the experiments described on page 26 of the specification and in the experiments described in my Declarations dated March 28, 2003 and November 28 2003 ("the later experiments"), several changes were made to our procedure that permitted us to detect subtle differences in chromatographic migration between Dh-OLF and dho-B. These differences in chromatographic migration can only be obtained if the structures of the mammalian compound (Dh-OLF) are different from the plant compound (dho-B).

(3) First, in the later experiments, we increased the sensitivity of detection by using a different antibody. In the experiments described in the specification, the antibody was raised against plant compounds dho-A and dho-B (*i.e.*, would bind to dho-A, dho-B and Dh-OLF). In the later experiments, the antibody was raised against only dho-B (*i.e.*, would bind mainly to dho-B and Dh-OLF, and much less so to dho-A). This increased antibody specificity allowed us to inject smaller quantities of Dh-OLF and dihydroouabain into the HPLC columns.

(4) Second, in the later experiments, we collected smaller elution fractions from the HPLC column [30 sec (0.5 mL) collection in the later experiments instead of 60 sec (1 mL) in

the experiments described in the specification]. Smaller collection volumes allows for detection of increased resolution in the separation. Thus, in the later experiments we detected two distinct peaks separated by a minimum of one minute (Figure 2 of November 28, 2003 Declaration), instead of one more-diffuse peak detected previously.

(5) Third, in order to further clarify that Dh-OLF (mammalian compound) was different from dho-B (plant compound), the two compounds were deglycosylation by acid hydrolysis to produce the genin-species. Because the genin-species are smaller, more fine resolution of chemical structures is possible. When we measured the HPLC mobility of the individual genin-species (Dh-OLF-genin and dho-B-genin), they eluted at 18 and 20 minutes respectively (see Figure 3 attached to Declaration dated November 28, 2003). Thus, the respective genins migrate differently for the two compounds. Therefore, they must have fundamentally different structures.

(6) It is also important to note that during the first experiments described in the specification, we never chromatographed the compounds together (mixed). In the later experiments, the four compounds (Dh-OLF, dho, Dh-OLF-genin, and dho-genin) were all run simultaneously in the same column, and generated four distinct peaks (See Figure attached to March 28, 2003 Declaration and Figure 3 attached to November 28, 2003 Declaration). Thus, subtle differences due to small variability between HPLC runs, rather than to differences in structure, were completely eliminated. Thus, Dh-OLF is structurally different than dho-B.

(7) It should be further noted that the reverse phase C-18 HPLC chromatography that we used separates compounds based primarily on their polarity and solubility properties. It is well established that many different compounds can migrate under one HPLC peak and have different structures, but have similar polarities and solubilities. The reverse, however, is definitive in that, if compounds separate, they must be different in structure, otherwise they would not have different polarities or solubilities.

(8) I hereby declare that all statements made herein are of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/2/04
Date

Roland Valdes, Jr., Ph.D

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3/28/03

S/N 09/503,559 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roland Valdes, Jr. et al.	Examiner: Ulrike Winkler
Serial No.:	09/503,559	Group Art Unit: 1648
Filed:	February 11, 2000	Docket: 1160.003US1
Title:	DIHYDROOUABAIN-LIKE FACTOR AND DIAGNOSTIC & THERAPEUTIC COMPOSITIONS AND METHODS	

DECLARATION OF DR. ROLAND VALDES, JR. UNDER 37 C.F.R. § 1.132

1. I, Roland Valdes, Jr., am one of the co-inventors of the above-identified patent application and am currently a Professor in the Department of Pathology and Laboratory Medicine at The University of Louisville in Louisville, Kentucky.

2. Physical properties related to molecular polarity and solubility can be distinguished by HPLC analysis. Thus, under identical experimental condition, a difference in chromatographic mobility is demonstrative of structural differences existing between the entities chromatographed.

3. In order to compare plant-derived dho and Dh-OLF, I performed the following experiments. Plant dihydroouabain (dho) and the mammalian Dihydro-OLF was isolated using standard purification techniques. The preparations were chromatographed both separately and mixed together under identical experimental conditions. The eluted HPLC fractions were measured using an antibody assay specific for the dihydro compounds.

4. Pure Dh-OLF was obtained from mammalian adrenal cortex (Qazzaz et al., Endocrinology, 2000;141(9):3200-3209) and pure Dho-B was obtained from HPLC separation of dihydroouabain commercial preparation (Qazzaz et al., Biochem Biophys Acta, 1999:1472:486-497). These experiments were performed with the purified intact molecules of dho and Dh-OLF and also after removal of the sugar moiety from each by hydrolysis. This approach tested both the intact molecule and the genin-form of each compound.

5. To remove the rahmonse moiety the parent molecules (Dh-OLF and Dho-B) were treated individually with 1% SSA for 45 seconds and the acid was immediately removed using a small C-

18 reversed-phase Sep-Pak solid-phase extraction cartridge column previously wetted with acetonitrile (AcN) and rinsed with deionized water. The Sep-Pak columns were eluted with 2 ml of 100% acetonitrile. To remove the AcN, the Sep-Pak eluents were evaporated to dryness in a Savant Speed Vac, dissolved the residue in 1 ml deionized water, and filtered the solution through a Whatman 0.22 μm PVDF filter in preparation for HPLC. The compounds were mixed together and co-injected on HPLC using an isocratic 10% CH_3CN mobile phase.

6. The two molecules, dho-B and Dh-OLF, separated by almost two minutes. Similarly, the genin compounds (aglycones, without sugar molecules) of both parents when mixed and injected on HPLC also separated by approximately 2 minutes on the same isocratic HPLC mode. The parent compounds, Dh-OLF and Dho-B eluted at 26 and 28, respectively. Their genin components (Dh-OLF-genin and dihydroouabain-B-genin) eluted at 18 and 20 mimutes, respectively (*See,* Attached Figure).

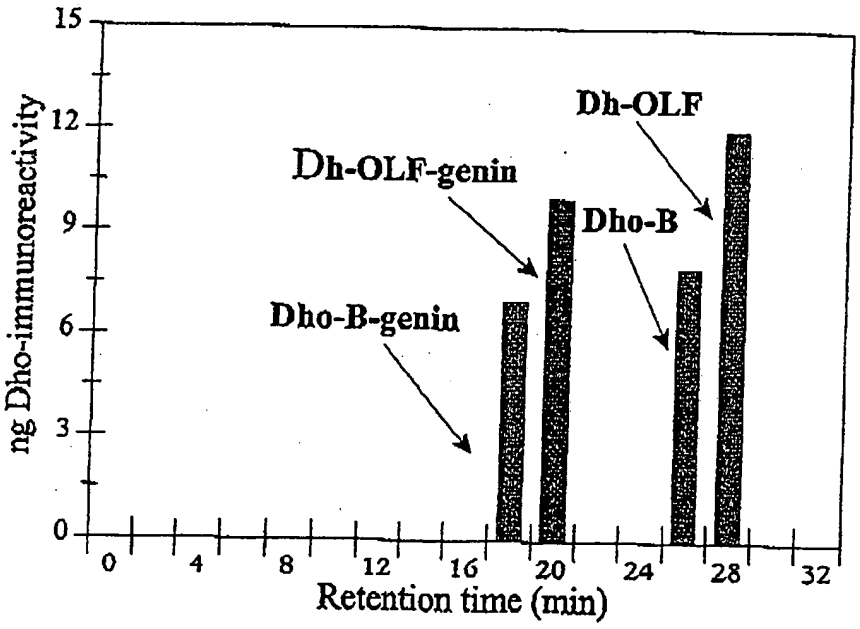
7. The separation of plant dihydroouabain from mammalian Dh-OLF and of dho-genin from Dh-OLF-genin by HPLC demonstrates conclusively that a structural difference exists between the plant dho and the Dh-OLF mammalian compounds.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

3/28/03
Date

Roland Valdes, Jr.

Chromatographic mobility of Dh-OLF and Dho-B and their genins



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S/N 09/503,559

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roland Valdes, Jr. et al.	Examiner: Ulrike Winkler
Serial No.:	09/503,559	Group Art Unit: 1648
Filed:	February 11, 2000	Docket: 1160.003US1
Title:	DIHYDROOUABAIN-LIKE FACTOR AND DIAGNOSTIC & THERAPEUTIC COMPOSITIONS AND METHODS	

SUPPLEMENTAL DECLARATION OF DR. ROLAND VALDES, JR. UNDER 37 C.F.R. § 1.132

1. I, Roland Valdes, Jr., am one of the co-inventors of the above-identified patent application and am currently a Professor in the Department of Pathology and Laboratory Medicine at The University of Louisville in Louisville, Kentucky.

2. Physical properties related to molecular polarity and solubility of lactone hydrogenated ouabain-like factor (Dh-OLF) isolated from different mammalian sources can be distinguished by HPLC analysis. Our chromatographic reverse-phase HPLC method uses an isocratic mode of 10% CH_3CN in H_2O to separate the Dh-OLF and dihydrouoabain-B entities with baseline resolution. This technique also separates the respective deglycolylated genins (aglycones). We report two important findings: 1) Dh-OLF isolated from human serum does not separate from Dh-OLF isolated from bovine adrenal glands; and 2) Dh-OLF isolated from human serum chromatographically separates from the plant-derived dihydroouabain isomer B (dho-B). These data demonstrate that Dh-OLF extracted form human serum is similar to that extracted from bovine adrenal glands and, of importance, that Dihydro-OLF is different from the plant-related counterpart, dihydroouabain (dho-B). These data strongly indicate that mammalian species make a common Dh-OLF.

3. Chromatographic co-migration of human serum Dh-OLF and bovine adrenal Dh-OLF. In these experiments purified Dh-OLF from humans serum and from bovine adrenal cortex were mixed together and co-injected on HPLC using an isocratic 10%

CH_3CN mobile phase. The two molecules showed a single band of elution time of 26 minutes (**Figure 1, attached**). This same HPLC technique has previously been demonstrated to separate Dh-OLFs with fine structural resolution (see also below). Thus, co-migration using these techniques is consistent with material from these two distinct mammalian sources being similar compounds.

4. Chromatographic separation of mammalian Dh-OLF from plant-derived dho compounds. Similarly, pure human serum Dh-OLF and bovine adrenal cortex Dh-OLF (Qazzaz et al., Endocrinology, 2000;141(9):3200-3209) and their plant related counterpart Dihydroouabain-isomer B (pure Dho-B obtained from HPLC separation of dihydroouabain commercial preparation, Qazzaz et al., Biochem Biophys Acta, 1999:1472:486-497) were mixed together and co-injected on HPLC using an isocratic 10% CH_3CN mobile phase. The three molecules showed two bands separated by a minimum of 1 minute (**Figure 2, attached**). The first band (26 min) was identified (see above) as a co-eluting mixture of the two sources of Dh-OLF (human serum and adrenal cortex, see **Figure 1, attached**) and the second band eluted at 28 min representing dho-B. Similarly, the genin compounds (aglycone without the sugar molecules) of both parents (human serum Dh-OLF and Dho-B) when mixed and injected on HPLC also clearly separated by 1 to 1.5 minutes using the same isocratic HPLC mode. While the parent compounds, human serum Dh-OLF and Dho-B eluted at 26 and 28 respectively those of their genin components (human serum Dh-OLF-genin and dihydroouabain-B-genin) eluted at 18 and 20 minutes respectively (**Figure 3, attached**). This demonstrates beyond doubt that the mammalian-derived Dh-OLF is chromatographically distinct from the plant-derived dihydroouabain.

5. Methods. To remove the rahmonse moiety we treated the parent molecules [Dh-OLF (both sources) and Dho-B] individually (separately) with 1% SSA for 15 seconds and immediately separated the acid using a small C-18 reversed-phase Sep-Pak solid-phase extraction cartridge column previously wetted with acetonitrile (ACN) and rinsed with deionized water. These columns were eluted with 2 ml of 100% acetonitrile. To

remove the ACN, we evaporated the Sep-Pak eluents to dryness in a Savant Speed Vac, dissolved the residue in 1 ml deionized water, and filtered the solution through a Whatman 0.22-μm PVDF filter in preparation for HPLC.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11/28/08
Date

Roland Valdes, Jr.

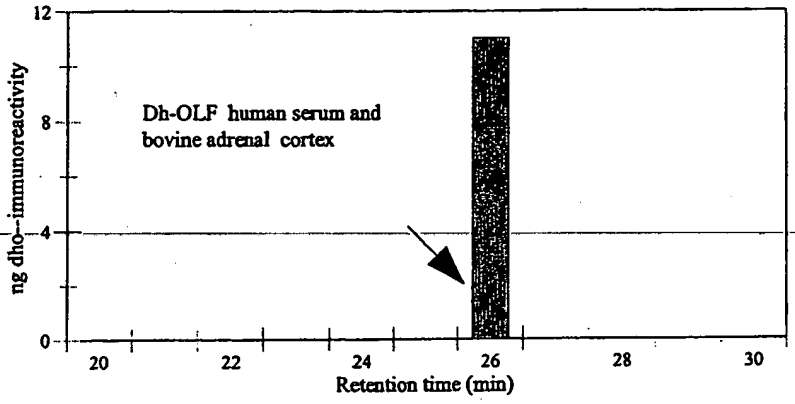

Figure 1. Chromatographic mobility of Dh-OLF isolated from human serum and bovine adrenal cortex mixed together

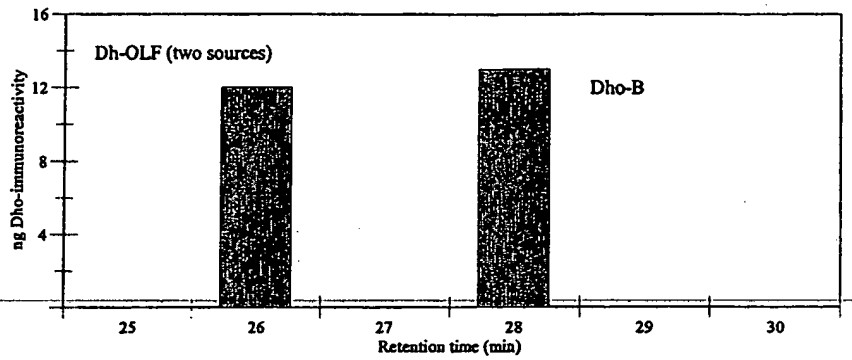

Figure 2. Chromatgraphic mobility of Dh-OLF (human serum) and Dh-OLF (adrenal Cortex) and plant related Dho-B

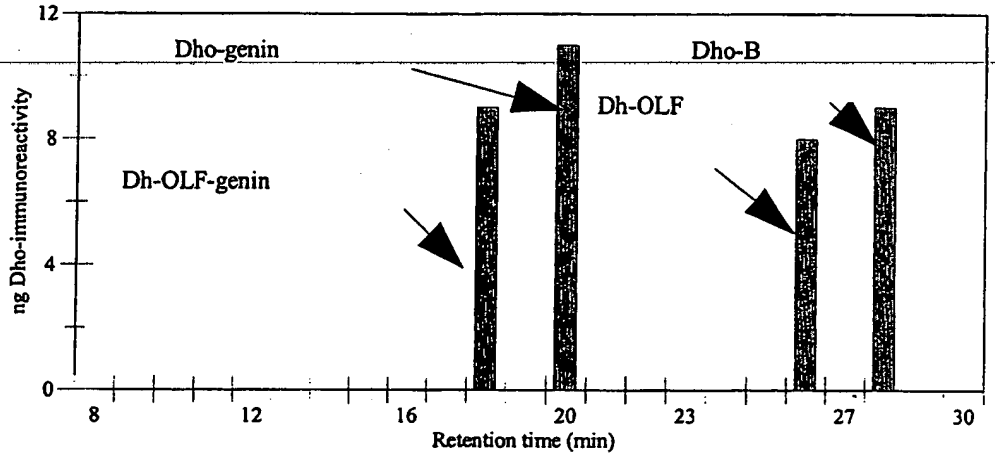

Figure 3. Chromatographic mobility of human serum Dh-OLF and dho-B and their congeners